Indirect Magnetic Force Microscopy - A Multimodal Technique

Thesis

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By

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Abstract

Magnetic micro- and nano-particles are widely used for labeling cells and tissues and for magneto-separation of biomolecules. Current approaches to detect these particles are limited to an ensemble of particles with poor spatial resolution at the single particle level. In this study we demonstrate how magnetism based microscopy can be utilized for detection of magnetic micro-particles in a label-free and multimodal manner.

Earlier work from our laboratory had demonstrated how the atomic force microscopy (AFM) based technique, magnetic force microscopy (MFM), can map magnetic nano-particles and ferritin protein at nanoscale resolution. MFM has thus far been performed using direct MFM (D-MFM), where multiple passes of the probe over the sample are required to identify magnetic interactions between the sample and probe. The purpose of this study was to employ a novel indirect MFM (ID-MFM) technique that requires only one pass over the sample, reduces possible probe contamination, and enables multi-modal microscopy. In ID-MFM, an ultrathin silicon nitride window, commonly used for transmission electron microscopy (TEM) is utilized as a barrier between the sample and the probe.

In this study ID-MFM was performed on commercially available paramagnetic and non-magnetic micro-particles. The paramagnetic micro-particles produced a distinct magnetic signature when imaged with the MFM probe as compared to an AFM probe. Non-magnetic particles produced no magnetic signature. All particles could be imaged with TEM and fluorescence microscopy as well, once mounted on the ID-MFM barrier, demonstrating the technique's multimodal capabilities. ID-MFM thus holds the promise to serve as a high-

throughput, ultrastructural, and multimodal technique for mapping micro- and nano-particles for a variety of applications.

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Chapter 1: Introduction

1.1 Direct Magnetic Force Microscopy and Its Limitations

Magnetic micro- and nano-particles are widely used to label cells[1] and tissues[2]. Techniques to detect such magnetic particles are thus important to better understand their specific labeling and distribution. Current techniques for detection of magnetic micro-particles primarily employ biochemical stains or magneto-sensors, which provide information on an ensemble of particles, with limited spatial resolution. While microscopy-based techniques can provide an improved spatial resolution at the single particle level, they are limited to chemical staining approaches or electron-dense detection in electron microscopy. One of the unique features of magnetic particles which has not been adequately exploited in microscopic analysis is their magnetic properties.

Magnetic force microscopy (MFM) is a high-resolution microscopy technique which is used to map magnetic domains in a sample. The current method used in MFM analysis is referred to as direct magnetic force microscopy (D-MFM) which is performed using a commercially available atomic force microscope (AFM). In D-MFM, a magnetically coated probe passes over the sample multiple times. First, the probe physically touches the sample to obtain an image of the topography. Then it passes over the sample again at various lift heights to determine the magnetic interactions between the sample and probe as well as to eliminate any confounding forces, such as electrical or van der Waals forces (Figure 1).

D-MFM is a widely used technique and has already been demonstrated as capable of mapping the magnetic signature of different types of samples, including memory storage devices, magnetic nano-particles[3, 4] and purified ferritin[5, 6]. Most recently, our laboratory has

demonstrated how D-MFM can be used to analyze iron rich lysosomes in mammalian tissue sections in a manner compatible with routine histological analysis[7,8].

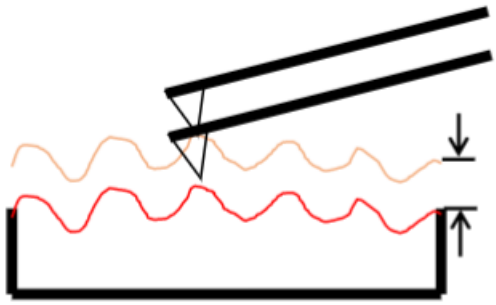

Figure 1: Schematic of D-MFM. The probe first makes one pass by physically touching the sample and another at a lift height above the sample.

However, several limitations exist within the scope of D-MFM. Having to make contact with the sample, the probe risks contamination and the multiple passes over the sample to obtain the magnetic signature are time consuming. Further the samples typically cannot be kept in a fluid environment in D-MFM.

1.2 Indirect Magnetic Force Microscopy and its Advantages

A novel technique developed in our laboratory is Indirect Magnetic Force Microscopy (ID-MFM). In this mode of MFM, the sample is separated from the magnetic probe by a nanoscale silicon nitride barrier (Figure 2). With the barrier in place, the probe is required to only make one pass over the sample to obtain the magnetic signature instead of taking multiple passes

at various lift heights above the sample. The barrier thus not only removes the possibility of contamination, but also creates more efficient experimentation with MFM.

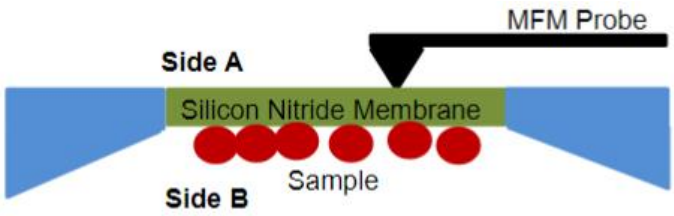

Figure 2: Schematic of ID-MFM. Indirect Magnetic MFM is performed on a silicon nitride membrane barrier separating a sample from the probe.

The barriers used can be commercially available TEM or X-ray diffraction windows. Because of this, ID-MFM has the potential to be high-throughput multimodal imaging technique that can be used to detect the magnetic signature of various types of samples.

1.3 Research Significance and Overview

The goal of this work was to examine the capabilities of ID-MFM as a multimodal tool for imaging magnetic micro-particles with the following objectives:

1. Test the capabilities of ID-MFM to map the magnetic signature of micro-particles.
2. Determine the multimodal capabilities of ID-MFM.

Chapter 2: Methodology

2.1 Sample Preparation

2.1.1 Micro-Particles

ID-MFM studies were performed using commercially available micro-particles. The particles used were yellow fluorescent carboxyl coated paramagnetic beads 2-2.4 μm in diameter from Spherotech, product number FCM 2052-2. These beads consist of a polystyrene core coated with a layer of iron oxide. As a control light yellow fluorescent non-magnetic micro-particles 1.7-2.2 μm in diameter were also used (product number FP 2045-2). Each type of particle was diluted with distilled water by a dilution factor of 50. The diluted solution was then vortexed for 30 seconds.

2.1.2 Silicon Nitride Membranes

Commercially available silicon nitride membranes commonly used in TEM studies were utilized for ID-MFM studies in this research. 20nm and 50nm thick membranes from SiMPore (product numbers SN100-A20Q05 and SN100-A50Q05, respectfully) as well as a 100nm membrane custom made from NIST were used for micro-particle studies. The SiMPore membranes themselves were 500x500 μm in size within a 100 μm wafer (Figure 3). The beads were immobilized on side B of Silicon Nitride membranes (Figure 2). To do so, the windows were carefully grasped with a pair of tweezers and flipped upside down. 2 μL of the diluted particle solution was then placed on top of side B of the membrane and allowed to dry. The membranes were then flipped back so that side A was facing up and placed onto a metal stub to

be imaged on side A with MFM or AFM probes. The presence of magnetic micro-particles on the membrane was verified by using fluorescence microscopy as well as TEM imaging. Phase and height images recorded with MFM or AFM probes were analyzed and compared to confirm the magnetic nature of the signal arising in ID-MFM.

Figure 3: Silicon Nitride Membrane. Example of a Silicon Nitride TEM grid from SiMPore, used as a barrier in ID-MFM for these studies.

2.2 Multimodal Imaging Techniques

2.2.1 Transmission Electron Microscopy

After ID-MFM analysis the very same silicon nitride TEM windows were analyzed using TEM. A JEM-1400 TEM (JEOL Ltd.) at 80 kV was used to image the samples. The TEM was equipped with a Veleta digital camera (Olympus Soft Imaging Solutions, GmbH).

2.2.2 Fluorescence Microscopy

Fluorescence microscopy was performed on the very same silicon nitride TEM windows to confirm the presence of fluorescent micro-particles on the membrane. A Zeiss Axiovert light microscope equipped with a 20X plan apochromat objective lens was used. Fluorescence microscopy was performed suing Semrock YFP-2477A filter with a 500/24 nm excitation, 542/27 nm emission, and FT 528 nm dichroic s. Membranes were placed on Fisherbrand glass cover slips to obtain the image.

2.3 ID-MFM

All AFM experiments were performed using a Multimode AFM equipped with a JV scanner and a Nanoscope IIIa controller (from Bruker). For ID-MFM studies, a high moment magnetic probe ASYMFMHM from Asylum Research was used. The probe was magnetized using an external magnet for 5 minutes prior to imaging. As a control non-magnetic AFM probes (NSC15) were used ID-MFM experiments. For ID-MFM no lift-mode imaging was employed and images were recorded on the top surface of the membrane. All AFM experiments were performed in tapping mode and the height and phase images were recorded. Images were recorded with 512 lines per scan direction using a scan speed of 1.8-3 Hz and a scan size of 5-20 μm. Drive amplitude, integral gain, and proportional gain were adjusted as needed for image clarity.

2.4 Image Section Analysis

The section analysis feature of the Nanoscope software was utilized for analysis of the ID-MFM images. The magnitude of the height and the diameter of the micro-particles was measured on the height image images and the phase signal was measured on the phase images. For the diameter analysis from the TEM and fluorescence microscopy images, the images were analyzed using ImageJ.

2.5 Statistical Analysis

A student's two-tailed unpaired t-test was performed to ascertain statistically significant differences between various parameters. A $p<0.05$ was regarded as significant.

Chapter 3: Results and Discussion

3.1 Multimodal Detection of Magnetic Micro-Particles

To determine the multimodal capabilities of ID-MFM, studies were performed on commercially available, fluorescently labeled paramagnetic micro-particles. Figure 4 shows fluorescence and TEM images of these particles immobilized on a 50 nm thick silicon-nitride TEM window. Quantitative analysis of particle size from these images revealed a particle size (diameter) of 1.38±.16 and 1.48±.20 μm, respectively.

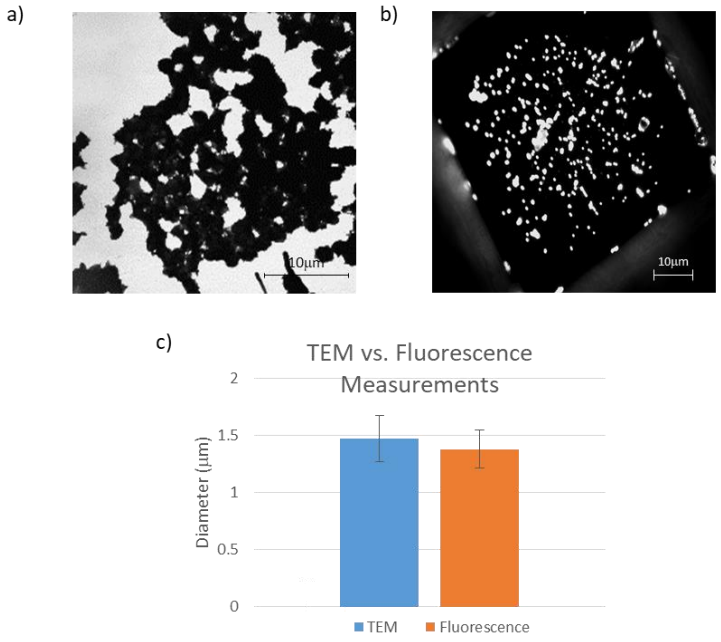

Figure 4: TEM and Fluorescence Microscopy Measurements. A comparison of particle diameter measured with the TEM and fluorescence microscopy techniques.

We next examined these particles by ID-MFM using both a MFM and an AFM probe. Although not expected in ID-MFM, the particles could be resolved even in topographic images

through the 50 nm thick membranes. As shown in Figure 5 section analysis was carried out on topographic images to ascertain particle size and height. No significant differences were observed between the particle diameter ascertained using topographic images in ID-MFM as compared to that obtained from fluorescence and TEM images ($p>.28$). Table 1 summarizes the particle size ascertained using fluorescence, TEM, AFM and MFM images.

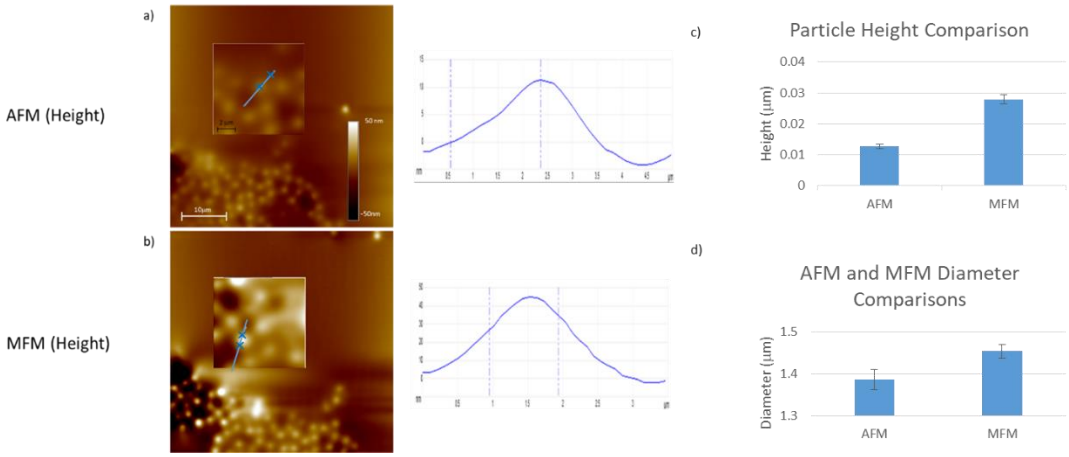

Figure 5: Paramagnetic Particle Height Analysis. Paramagnetic particles imaged with an a) AFM probe and a b) MFM probe. Section profiles, measured for an individual particle, are shown next to their corresponding image. Comparison of the c) height and d) diameter taken with the two probes is shown graphically.

To ascertain if the particle topography observed in ID-MFM corresponded to the expected height of particles and thus meant that the particles were on the exposed surface A of the membrane, we also examined the topographic height of the particles in ID MFM images. The particle height in using both AFM and MFM probes was < 50 nm (Figure 5 and Table 1), indicating that the observed topographic effects were minor.

Table 1: Multimodal Measurements of Paramagnetic Particles. Average diameter and height measurements taken with various microscopy techniques.

	Diameter (μm)	Height (μm)
Fluorescence	1.38±.16	NA
TEM	1.48±.20	NA
ID-MFM (AFM probe)	1.39±.02	.01278±.00074
ID-MFM (MFM probe)	1.45±.03	.02793±.00151

3.2 Distinguishing Magnetic vs. Nonmagnetic Particles

To examine if ID-MFM can identify the paramagnetic micro-particles from non-magnetic particles, we employed two approaches (a) analyzed paramagnetic particles using a MFM and an AFM probe and (b) analyzed paramagnetic and non-magnetic particles using a MFM probe.

Figure 6 shows height and phase images of paramagnetic particles imaged using a MFM and an AFM probe. Quantitative analysis of the magnitude of maximum phase shifts showed a significantly higher magnitude of phase shift obtained using MFM probe (Table 2).

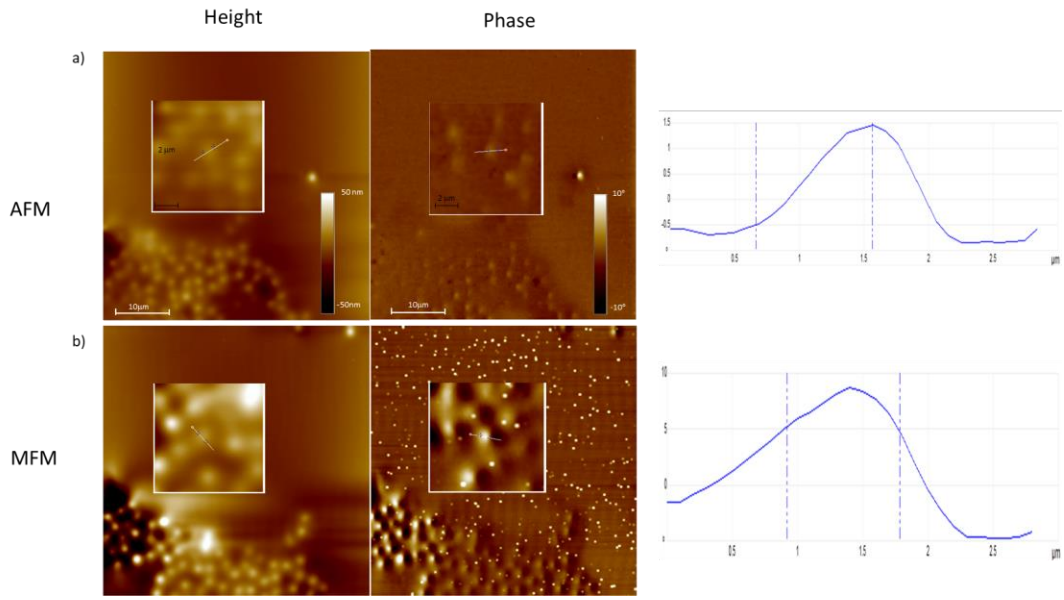

Figure 6: Paramagnetic Particle Phase Analysis. Height and phase images of paramagnetic particles taken with an a) AFM and b) MFM probe as indicated with corresponding section analysis profile of the phase image.

We next compared paramagnetic vs. non-magnetic micro-particles using a MFM probe (Figure 7, Table 2). As expected, non-magnetic particles experienced virtually no phase signal. While a very faint signal could be detected by eye, the actual value of this phase signal could not be detected through section analysis techniques. Height images taken of non-magnetic particles with both the AFM and MFM probe appeared similar to those taken of paramagnetic particles.

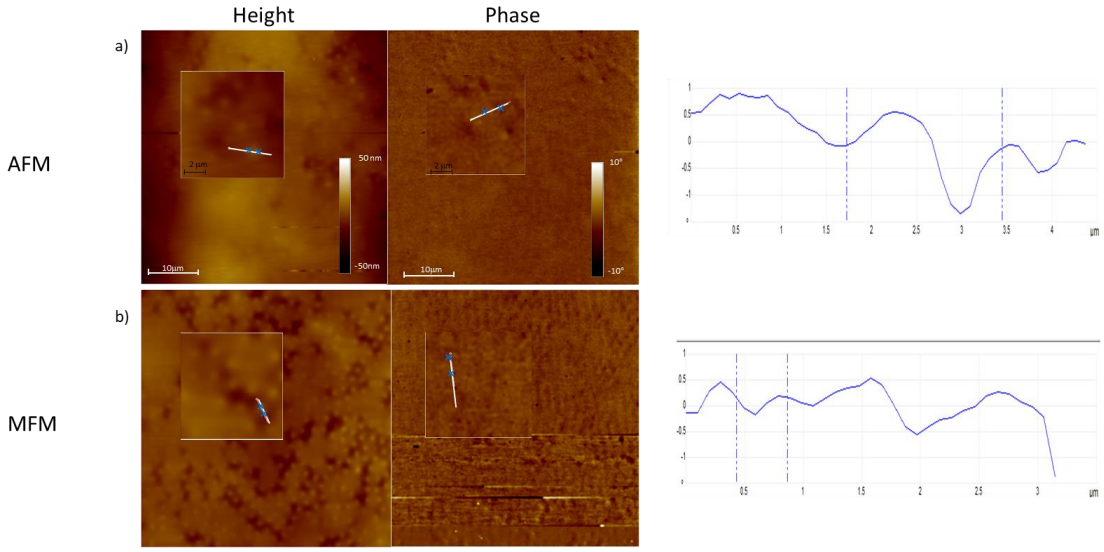

Figure 7: Non-Magnetic Micro-Particle Imaging. This figure shows the height, phase and corresponding section analysis of the phase image for a) AFM and b) MFM of non-magnetic micro-particles.

Table 2: Phase Measurement Comparisons. The magnitude of the phase signal obtained with an AFM and MFM probe of the two types of particles.

Particle Type	Probe Type	Phase (°)
Paramagnetic Particles	AFM	.81±.03
	MFM	12.81±.57
Non-Magnetic Particles	AFM	No distinct signal detected
	MFM	No distinct signal detected

Further when paramagnetic particles were evaluated for different membrane thicknesses, the AFM and MFM signals were not as clear. For 20nm membranes, signals exhibited significant noise and no distinct particle signal (data not shown). This is likely because the membrane, being so thin, was more easily deformed by the relatively larger micro-particles and thus the probe

could not make a pass over the sample efficiently. For 100nm membranes, no particle signal was detected, most likely because the membrane was too thick (Figure 8). Because no signal was detected with MFM, no AFM studies were performed on the 100nm membrane. This demonstrates how membrane thickness must be optimized for various samples. This optimization may be able to be determined in conjunction with D-MFM studies on the samples. As the lift height is raised in D-MFM, the phase signal adjusts. An optimal lift height may correspond with an optimal membrane thickness.

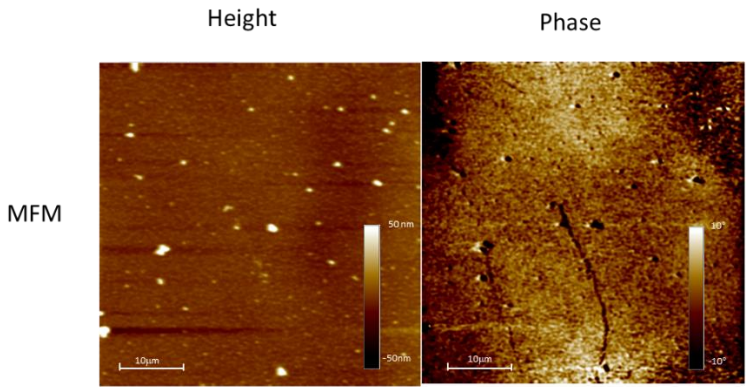

Figure 8: Micro-Particle Images with a 100nm Membrane. Micro-particles imaged on 100nm membrane. No clear signal was obtained.

Our results thus elucidate that ID-MFM was capable of detecting the magnetic micro-particles albeit with some topography cross-talk. We postulate that this topographic effect is likely due to local changes in mechanical (or other) properties of the membrane where the particles are present and impacts the cantilever oscillation in the tapping mode. It is also possible

that the micro-particles, being much larger in diameter in comparison to the thickness of the membrane, deformed the membrane to create nanoscale effects in topography.

Chapter 4: Conclusion

This work identified the capabilities and limitations of ID-MFM as well as laid the groundwork for further studies to be performed. ID-MFM was successfully utilized as a multimodal microscopy method for characterizing magnetic micro-particles. Use of 50nm thick membranes provided a barrier between the probe and the sample to eliminate confounding signals while still detecting and imaging the magnetic signature of the particles. The immobilized particles could also be imaged using TEM and fluorescence microscopy. ID-MFM of paramagnetic micro-particles showed a distinct magnetic signal compared with non-magnetic micro-particles and when using the non-magnetic AFM probe.

One of the unexpected results from of ID-MFM was that sample topography could still be detected to some extent in ID-MFM. While this was advantageous to localize the particles and confirm their presence on the membranes, it does indicate that ID-MFM is not completely free from artifacts. It is interesting to note that the topography effect is minimized when using thicker membranes but at the compromise of ID-MFM signal. Thus an optimal membrane thickness may be required for various sample types.

The use of silicon nitride windows has thus far been limited to electron microscopy[11] or x-ray optics and diffraction[12]. One study has previously demonstrated how magnetic micro-particles could be manipulated across a 100 nm thick window through the use of sanded MFM probes[13]. Our study demonstrates how ID-MFM can serve as multimodal imaging tool to detect magnetic particles. Further research could help understand the full capabilities and limitations of ID-MFM for both biological as well as non-biological samples.

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